Agents with Selective Estrogen Receptor (ER) Modulator Activity Induce Apoptosis

In vitro and In vivo in ER-Negative Glioma Cells

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ABSTRACT

Tamoxifen, a member of the selective estrogen receptor modulator (SERM) family, is widely used in the treatment of estrogen receptor (ER)-expressing breast cancer. It has previously been shown that high-dose tamoxifen has cytotoxic activity against glioma cells, but whether this effect is drug specific or represents a general property of SERMs is unknown. In this study, we demonstrate that tamoxifen and CC-8490, a novel benzopyranone with SERM activity, induce glioma cell apoptosis in a dose- and time-dependent manner. Moreover, administration of tamoxifen and CC-8490 suppresses tumor growth in vivo and extends animal survival in glioma xenograft models. None of the eight glioma cell lines examined express either ER-α or -β, suggesting the mechanism for tamoxifen– and CC-8490–induced glioma cell apoptosis is independent of the ER signaling pathway. Complementary DNA microarray expression profiling allowed us to identify a subset of genes specifically regulated by tamoxifen and CC-8490, and not by other apoptotic stimuli, including nuclear factor (NF)-κB with its target genes IEX-3, SQD2, IL6, and IL8. We demonstrate that suppression of NF-κB activation markedly enhances SERM-induced apoptosis, suggesting a role for NF-κB in protecting glioma cells from SERM-induced cytotoxicity. These findings demonstrate for the first time that a SERM other than tamoxifen can induce glioma cell apoptosis in vitro and in vivo and that the clinical efficacy of SERMs for the treatment of malignant gliomas could potentially be enhanced by simultaneous inhibition of the NF-κB pathway.

INTRODUCTION

Gliomas are the most common type of primary brain tumor and one of the most aggressive of all malignancies. The prognosis for patients with malignant gliomas has remained largely unchanged over the last two decades, with median survivals of less than a year for patient with glioblastomas, the most common and aggressive type of malignant glioma. Clearly, new and effective therapies are desperately needed.

Tamoxifen, a member of the selective estrogen receptor modulator (SERM) family, is widely used in the treatment and prevention of breast cancer. Because antitumor effects have been predominantly observed in patients with estrogen receptor (ER)-positive tumors, it is generally accepted that the primary actions of tamoxifen are mediated through inhibition of the ER pathway. A demonstration that some ER-negative breast cancers also respond to tamoxifen, however, has suggested the possibility of an ER-independent antitumor mechanism and led to the use of tamoxifen in the treatment of other types of cancer (1, 2). Although therapeutic activity of tamoxifen alone or in combination with other agents has been demonstrated in some ER-negative cancers, the mechanism of action of tamoxifen in these circumstances remains largely unknown (3).

Tamoxifen has been shown to inhibit glioma cell proliferation and induce apoptosis in vitro, an effect associated with inhibition of protein kinase C (PKC) activity (4, 5). These preclinical studies provided the impetus for clinical trials of tamoxifen in patients with malignant gliomas. Treatment of patients with recurrent malignant gliomas with low-dose tamoxifen revealed minimal activity, although trials using high-dose tamoxifen alone or in combination with other cytotoxic agents have demonstrated some activity. Nevertheless, response rates and overall patient outcome were far from optimal (6–10). Given the excellent tolerability, nonoverlapping toxicities, and non-cross-resistance to standard DNA-damaging chemotherapeutic agents, SERMs represent a theoretically promising therapy for gliomas if their antitumor activity can be improved. For the purposes of future antglioma drug development, the question remains whether the antglioma effects of tamoxifen are drug specific or represent a general property of this class of drugs. Thus, the major aim of our studies was to better understand the action of tamoxifen on malignant gliomas by attempting to identify SERMs with more potent antglioma activity. We demonstrate for the first time that CC-8490, a benzopyranone with SERM activity (11), has antglioma activity in vitro and in vivo and that the antglioma activity of both tamoxifen and CC-8490 can be accentuated by inhibiting the nuclear factor (NF)-κB pathway.

MATERIALS AND METHODS

Cells and Reagents. Human glioma cell lines U87MG, T98G, U251MG, U373MG, U138MG, WS1088, A172, and N1321N1 were obtained from American Type Culture Collection (Manassas, VA). U87MG, T98G, and U138MG, U251MG, and U373MG were maintained in minimal essential medium (Invitrogen, Carlsbad, CA), A172 and 1321N1 were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen), and WS1088 was maintained in Leibovitz’s L-15 medium (Cellgro, Herndon, VA), supplemented with 10% fetal bovine serum. Tamoxifen, CC-8490, and Faslodex were obtained from Celgene Corp. (Warren, NJ). CC-8490 is a new benzopyranone with a molecular weight of 586.05 and the following backbone structure (Scheme 1).

Details of the general approach to chemical synthesis of CC-8490 can be found in ref. 11, and more information on this series of compounds can be found in United States Patent 6,291,456 B1.2 The vehicle for CC-8490 and tamoxifen is 0.1% dimethyl sulfoxide (DMSO). 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU), Taxol, and salicylate were purchased from Sigma (St. Louis, MO).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyldiazotetrazolium Bromide Assay. U87MG, U138MG, and U373MG cells were seeded in 96-well plates at a concentration of 5 × 103 cells per well. At 24 hours after seeding, cells were continuously exposed to tamoxifen or CC-8490 at different concentrations (5, 10, 20, 40, and 80 μmol/L) or vehicle (0.1% DMSO) for 48 hours. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyldiazotetrazolium bromide (MTT) assay was performed using Cell Proliferation Kit I (Roche, Indianapolis, IN) according to the manufacturer’s manual. The assay was done in triplicate for each data point.

Apoptosis Assay by Terminal Deoxynucleotidyl Transferase-Meditated Nick End Labeling and 4’,6-Diamidino-2-phenylindole Staining. U87MG cells were plated in Lab-Tek II chamber slides suitable for tissue cultures (Nalge Nunc, Naperville, IL) at a concentration of 5 × 105 cells per well. Twenty-four hours after seeding, cells were exposed to 20 and 40 μmol/L tamoxifen or CC-8490 or 0.1% DMSO for 6, 24, or 48 hours. After treatment,
cells were fixed in 1% paraformaldehyde, and apoptosis was detected by the terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) method using the ApopTag Red In Situ Apoptosis Detection Kit (Intergen, Purchase, NY) as per the manufacturer’s instruction. Counterstaining with 4’,6-diamidino-2-phenylindole [DAPI (1 μg of DAPI/mL in PBS)] was performed to observe the nuclear morphology.

Quantification of Apoptosis by Flow Cytometry. U87MG cells were treated with 10, 20, and 40 μmol/L tamoxifen or CC-8490 or 0.1% DMSO for 6, 24, or 48 hours, and then adherent cells were harvested with 0.1% EDTA together with detached cells. DNA was stained with 50 μg/mL propidium iodide and protected from light before analysis with a FACScalibur (Becton Dickinson, Franklin Lakes, NJ). DNA histograms were interpreted using Cell Quest and ModFit software (Verity Software House, Topsham, ME).

Intracellular Calcium Measurement. U87MG cells were seeded at a concentration of 10^4 cells per well in a 96-well plate. Twenty-four hours after seeding, cells were exposed to 20 μmol/L tamoxifen, CC-8490, Faslodex, or 0.1% DMSO for 2, 4, 8, 16, or 24 hours. After treatments, cells were loaded in culture medium containing 5 μmol/L Fluo-4 AM and incubated at 37°C for 1 hour. Before measurement, cells were washed with Hanks’ balanced salt solution (HBSS) and then stimulated with 5 μmol/L calcimycin. Fluorescent intensity was measured using Vector 1420 Multilabel Counter (Perkin-Elmer, Wellesley, MA) with a 485/535 (excitation/emission) filter set.

Protein Kinase C Activity Assay. U87MG cells were treated with 5, 20, 40, and 60 μmol/L tamoxifen or CC-8490 or 0.1% DMSO for 18 hours. After treatment, cells were lysed in M-PER mammalian Protein Extraction reagent (Pierce Rockford, IL). PKC activity was measured using IQ PKC Assay Kit (Pierce). Enzyme reaction mix was incubated for 1 hour at room temperature. A no-enzyme sample was included as blank reference, and 1:9 diluted purified PKC enzyme standard (Pierce) was used as a positive control. The fluorescent intensity was measured using Vector 1420 Multilabel Counter with a 590/560 (excitation/emission) filter set.

Complementary DNA Microarray Analysis. U87MG cells were treated with 10 and 40 μmol/L tamoxifen or CC-8490 or 0.1% DMSO for 6 or 18 hours. After treatments, total RNA was isolated from U87MG cells using TRIzol (Life Technologies, Inc., Carlsbad, CA). A pool of an equal mixture of RNA from 13 human cell lines was used as reference. The cDNA probes were generated from total RNA with cy5- or cy3-dUTP (Amersham Pharmacia, Piscataway, NJ). The probes synthesized from the treatment samples and their corresponding controls were hybridized side-by-side with two identical microarray National Cancer Institute core “OncoChips,” which contain 9,980 features. All experiments were done in duplicate, and the reproducibility was confirmed by Pearson correlation analysis.

Real-Time Reverse Transcription-Polymerase Chain Reaction. U87MG cells were treated with 40 μmol/L CC-8490 for 18 hours, and total RNA was isolated using TRIzol. First-strand cDNA was reverse transcribed from total RNA using a random hexadeoxynucleotide primer. All polymerase chain reaction (PCR) primers and TaqMan probes were designed using Primer Express software (Applied Biosystems, Foster City, CA) using published sequence from the National Center for Biotechnology Information database. Primers and TaqMan probes were purchased from Applied Biosystems and labeled with the reporter dye FAM (6-carboxy fluorescein) in the 5’ end. All reactions were performed in triplicate using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems).

Estrogen Receptor-α and -β Expression in Glioma Cells. ER-α and ER-β expression in glioma cells (U87MG, T98G, U251MG, U373MG, U138MG, WS1088, A172, and N121H1) was determined by conventional reverse transcription-polymerase chain reaction. First-strand cDNA was prepared from total cellular RNA using a random hexamethoxynucleotide and SuperScript RNAase H reverse transcriptase (Invitrogen). ER-α mRNA was amplified using primers 5’-TACTGCATCAGATCCAAGGG-3’ (sense) and 5’-ATCAATGGTGCACTGGTG-3’ (antisense), ER-β mRNA was amplified using primers 5’-TGTAAGGAGGTAGTTGGAAACC-3’ (sense) and 5’-TGTTGACGAGGACATCATCAGGG-3’ (antisense) and, as a control, β-actin mRNA was amplified with 5’-CGAGCCGGGAAATCGTGTTGACAT- TAAAGGA-3’ (sense) and 5’-GCTGATCTCTGCTGTGATCCA- CATCTGC-3’ (antisense). Primer pairs for each gene spanned at least one intron to distinguish amplified cDNA products from genomic DNA. The PCR cycle parameters were as follows: 94°C for 60 seconds, 55°C for 60 seconds, and 72°C for 60 seconds for ER-α and β-actin, and 94°C for 60 seconds, 58°C for 60 seconds, and 72°C for 60 seconds for ER-β. The reaction products were separated electrophoretically on a 3% agarose gel and stained with ethidium bromide for visual evaluation.

Nuclear Factor-κB Activity Assay. Enzyme-linked immunosorbent assay (ELISA) was used to test NF-κB activity. U87MG cells were seeded onto 6-well plates, and 24 hours later, the cells were treated with 40 μmol/L tamoxifen, 40 μmol/L CC-8490, or 0.1% DMSO. For salicylate pretreatment experiments, U87MG cells were pretreated with 10 mmol/L salicylate, and 1 hour later, they were exposed to 40 μmol/L tamoxifen or 40 μmol/L CC-8490. Cell lysates were collected 8 hours after initiating tamoxifen or CC-8490 treatments. Three micrograms of cell extract were placed into each well of 96-well ELISA plates. One hundred microliters of diluted NF-κB antibody (1:10,000 dilution) were added into each well and then incubated at room temperature for 1 hour. Horseradish peroxidase-conjugated antirabbit IgG was used as second antibody at a dilution of 1:10,000. After colorimetric reaction, absorbance was read on a spectrometer at 490 nm.

Subcutaneous Tumor Model. Eighteen 4- to 6-week-old athymic nude mice were used for the experiments. U87MG cells (2 × 10^6) in 100 μL of HBSS were injected into the right posterior flank of the mice. At day 3 after tumor inoculation, the mice were treated with intraperitoneal injection of tamoxifen (30 mg/kg/d; n = 6), CC-8490 (30 mg/kg/d; n = 6), or vehicle (n = 6). The mice tolerated CC-8490 and tamoxifen extremely well, as assessed by maintenance of high activity level, continued weight gain, and good grooming, until the time they began to develop neurologic symptoms attributable to tumor growth. There were no CC-8490–related animal deaths. The mice were treated for 28 days and then sacrificed. Tumors were measured and weighed. In situ apoptotic cell detection was performed using ApopTag Peroxidase Kit (Intergen). The apoptosis index was calculated by assessing the percentage of ApopTag-positive cells in five high-power microscopic fields (>400) for each specimen.

Intracranial Tumor Model. The mouse intracranial tumor model was established as described previously (12). In brief, 10^6 U87MG cells were suspended in 5 μL of HBSS and injected into the right caudate nucleus of athymic nude mice (age, 4–6 weeks). Twenty-four mice were divided into three groups (n = 8 for each group) and treated daily with intraperitoneal injection of tamoxifen (30 mg/kg), CC-8490 (30 mg/kg), or vehicle starting 3 days after tumor inoculation. For the intracerebral convection drug delivery, we first performed a preliminary toxicity experiment in which 10, 50, 500, 1,000, and 5,000 μmol/L CC-8490 were convected into the normal brain of non–tumor-bearing nude rats (n = 3 for each concentration). Convolution at these concentrations caused no animal deaths or clinical signs of toxicity for up to 7 days after convolution. The rats were sacrificed at day 7 after convolution, and histologic examination revealed no signs of neurotoxicity in the areas of convected brain tissue. To establish a xenograft tumor, 10^6 U87MG cells in 5 μL of HBSS were injected into the right caudate nucleus of nude rat (age, 4–6 weeks). Twelve rats were divided into two groups (n = 6 for each group), and 1 mmol/L CC-8490 or vehicle (10 μL over 20 minutes) was convected into tumor using the same stereotactic coordinates starting a day after tumor implantation.

Statistical Analysis. Student’s t test was used to determine the significance between different groups. Survival curves were plotted using the Kaplan-Meier method and compared using the log-rank test. P < 0.05 was considered statistically significant.

RESULTS

Selective Estrogen Receptor Modulators Induce Apoptosis In vitro. Fig. 1 demonstrates that tamoxifen and CC-8490 caused dose-dependent growth inhibition in glioma cell lines U87MG, U138MG, and U373MG. By contrast, Faslodex, a pure ER antagonist, did not
suppress glioma cell growth (Fig. 1A). Glioma cell exposure to SERMs caused cell shrinkage, nuclear condensation, and fragmentation, indicative of apoptosis. The SERM-induced morphologic changes were dose and time dependent (Fig. 1B). The presence of apoptotic cell death was confirmed by DAPI staining and TUNEL assay (Fig. 1C). Consistent with the TUNEL assays, fluorescence-activated cell-sorting analyses revealed that SERMs induced a sub-G0-G1 population of cells in a dose- and time-dependent manner. For
example, glioma cells exposed to 40 μmol/L SERMs for 6, 24, and 48 hours demonstrated an increase in the sub-G₁-G₀ population of cells from 5% to 50% to >80% (Fig. 1D).

Selective Estrogen Receptor Modulators Inhibit Tumor Growth In vivo. We then investigated the effects of tamoxifen and CC-8490 in a malignant glioma xenograft model in vivo. In a subcutaneous tumor model, peritoneal injection of SERMs significantly suppressed tumor growth as determined by tumor weight and volume (Fig. 2A). TUNEL assay revealed that SERMs markedly sensitized glioma cells to apoptosis in vivo; apoptosis index was 2.9 ± 1.4% in the tamoxifen-treated group, 3.1 ± 1.6% in the CC-8490-treated group, and 1.1 ± 0.7% in the vehicle-treated group (tamoxifen versus vehicle, \( P < 0.001 \); CC-8490 versus vehicle, \( P < 0.001 \); Fig. 2A). All mice bearing intracranial tumors treated with the vehicle control died within 4 weeks after tumor inoculation, whereas treatment with the SERMs significantly improved animal survival (Fig. 2B; tamoxifen versus vehicle, \( P < 0.01 \); CC-8490 versus vehicle, \( P < 0.01 \)). Direct intracerebral administration of the vehicle control or CC-8490 via a clinically used methodology known as convection-enhanced delivery (ref. 13) enhanced the antitumor effects of CC-8490, curing 50% of the drug-treated animals (Fig. 2C; \( P < 0.005 \)).

Glioma Cells Do Not Express Estrogen Receptor-α and β. To confirm that the antiglial effect of the tamoxifen and CC-8490 was not mediated through the ER, we evaluated glioma cell lines for the presence of ER. None of the eight human glioma cell lines examined using reverse transcription-PCR expressed either ER-α or ER-β. By contrast, ER-α and ER-β expression was observed in MCF-7 breast and PC-3 prostate cancer cells, respectively (ref. 14; Fig. 3). RNA protection assays in two glioma cell lines provided further evidence for the absence of ER-α or -β expression (data not shown). These data suggest that tamoxifen and CC-8490 must induce apoptosis in glioma cells through an ER-independent pathway, thus leading us to explore other signaling pathways that might play a role in SERM-induced glioma cell apoptosis.

Tamoxifen and CC-8490 Induce Intracellular Calcium Influx but Do Not Inhibit Protein Kinase C Activity. It has been suggested that one of the mechanisms by which tamoxifen induces apoptosis in glioma cells is related to inhibition of PKC activity. In this study, we found no difference in PKC activity between cells treated with vehicle (0.1% DMSO) and those treated with variable concentrations of tamoxifen or CC-8490 (Fig. 4A). By contrast, tamoxifen and CC-8490 induced a significant increase in cytosolic calcium concentrations consistent with previous observations (Fig. 4B; ref. 5). Calcium influx, however, was not induced by Faslodex, a pure ER antagonist that does not kill glioma cells (Figs. 1A and 4B).

Gene Expression in Glioblastoma Cells Treated with CC-8490 and Tamoxifen. To better understand mechanisms that may be operative in SERM-induced glioma cell apoptosis, we evaluated the gene expression profiles of glioma cells exposed to vehicle control or low-dose (10 μmol/L) or high-dose (40 μmol/L) tamoxifen or CC-8490 after 6 or 18 hours of drug exposure. An 18-hour exposure of U87MG cells to either 40 μmol/L tamoxifen or 40 μmol/L CC-8490 resulted in a statistically relevant 2-fold up- or down-regulation (at a \( P \) of <0.05) of 127 and 73 genes, respectively. Thirty-three of these genes were found to be common to both the tamoxifen- and the CC-8490–treated glioma cells (Table 1). Twenty-six of these genes were induced and seven genes were suppressed after exposure to tamoxifen and CC-8490. Interestingly, 15 of the 27 known genes are known to be associated with cell death or cell cycle functions.

To determine whether the genes identified as being deregulated after glioma cell exposure to tamoxifen and CC-8490 were specific to...
that were uniquely associated with exposure to CC-8490 and tamoxifen. Thus, there were 20 genes that showed aberrant expression patterns.

SERM-induced glioma gene expression generally occurred only under conditions (times and concentrations) that were associated with a lack of effect on cell viability at this concentration.

The results, taken together, demonstrate that significant changes in a SERM-induced apoptotic mechanism or merely a general consequence of apoptotic cell death, we compared the SERM-induced expression profiles with those obtained after treatment of U87MG cells with BCNU (250 μmol/L), Taxol (25 μmol/L), or serum starvation, conditions that also cause apoptosis in the U87MG cells (data not shown). Of the 33 SERM-responsive genes, 13 genes were also found in cells exposed to the other apoptotic stimuli (e.g., GADD45A). Thus, there were 20 genes that showed aberrant expression patterns that were uniquely associated with exposure to CC-8490 and tamoxifen (Table 1). These genes included NF-κB1, IEX-1 (also known as IER3), and SOD2.

Microarray analysis of mRNA from U87MG cells exposed to 40 μmol/L tamoxifen, 40 μmol/L CC-8490, or vehicle for only 6 hours demonstrated deregulated expression in six common genes including ATF3, DSCR1, RNAHP, SES2, and SLC3A2 (up-regulated) and SNK (down-regulated). A lower dose of CC-8490 and tamoxifen (10 μmol/L) had little impact on gene expression in U87MG cells, consistent with a lack of effect on cell viability at this concentration. These results, taken together, demonstrate that significant changes in SERM-induced glioma gene expression generally occurred only under conditions (times and concentrations) that were associated with in vitro cytotoxicity. This suggests that at least some of the gene expression changes may be associated with SERM-induced cell death.

Confirmation of Results from Complementary DNA Microarray by Real-Time Polymerase Chain Reaction. We were interested in exploring the significance of the finding that expression of the NF-κB1 gene and its downstream target gene, IEX-1, were deregulated by SERM-induced apoptosis, but not by other apoptotic stimuli, in light of our observation that several other SERM-responsive genes (SOD2, IL6, and IL8) have also been previously associated with the NF-κB pathway. These led us to hypothesize that the NF-κB pathway might be involved in SERM-induced apoptosis. To test this hypothesis, we first verified the reliability of the microarray data. Consistent with these data, real-time, quantitative PCR showed that tamoxifen- and CC-8490–induced apoptosis resulted in the up-regulation of NF-κB1 (4-fold increase) and other NF-κB pathway-related genes including IEX-1 (6-fold increase), SOD2 (19-fold increase), IL6 (8-fold increase), and IL8 (6-fold increase; Fig. 5). We additionally confirmed the changes in expression of several other apoptosis-associated genes such as GADD45A (13-fold increase), DDI73 (44-fold increase), and DSCR1 (23-fold increase) that were up-regulated by both tamoxifen and CC-8490 as well as by other cytotoxic stimuli (BCNU, Taxol, and serum starvation).

Nuclear Factor-κB Activation Plays an Antiapoptotic Role in Selective Estrogen Receptor Modulator-Induced Cell Death. In addition to the changes in gene expression, we evaluated the level of NF-κB activity in glioma cells after exposure to tamoxifen and CC-8490. ELISA assays demonstrated that tamoxifen– and CC-8490–induced apoptosis resulted in a 4-fold increase in NF-κB activity in glioma cells relative to the vehicle-treated cells (Fig. 6A). Although the data were highly suggestive that NF-κB was perturbed by exposure to the drugs, it was unclear what role, if any, NF-κB was playing in drug-induced cytotoxicity. It has been reported previously

![Graph](image)

**Table 1** SERM-responsive genes

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<th>Name</th>
<th>Descriptions</th>
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* Genes also regulated by BCNU or/and Taxol or/and starvation.

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that NF-κB can play a context-dependent dual antiapoptotic and proapoptotic role, depending on the nature of stimuli and the tissue type. Therefore, to better define the role of NF-κB activation in SERM-induced apoptosis, we inhibited NF-κB activity in U87MG cells by preexposing the cells to salicylates, a known NF-κB inhibitor, before challenging the cells with SERMs. NF-κB activity was significantly suppressed by pretreatment with salicylates (Fig. 6A). Additionally, pretreatment of U87MG cells with 5 and 10 mmol/L of salicylates markedly enhanced tamoxifen- and CC-8490–induced cell death (Fig. 6B-I, B-II, and C). By contrast, exposure of glioma cells to salicylates alone at concentrations ranging from 5 to 10 mmol/L did not significantly affect cell viability (Fig. 6B-III).

**DISCUSSION**

Tamoxifen has been the prototypic hormonal therapy for breast cancer more than two decades. The efficacy of tamoxifen is most pronounced in breast cancers that express the ER, consistent with the known function of tamoxifen as an ER agonist/antagonist. Thus, it is well established that the principle antitumor mechanism of tamoxifen in breast cancer is through the ER signaling pathway. Nevertheless, it has been demonstrated that tamoxifen can exert an antitumor effect in ER-negative breast cancer and other cancers. The mechanism for this ER-independent antitumor effect remains largely unknown.

Several investigators have reported that tamoxifen has antiglioma activity in vitro, and several small, single-institution trials documented some minimal activity in patients with recurrent gliomas. In fact, tamoxifen is often given to patients with recurrent malignant gliomas as a last resort when all other therapies have failed, given its ease of administration and minimal toxicity profile. These characteristics would make tamoxifen a theoretically attractive treatment option for malignant gliomas if the relatively small responses rate could be improved. A better understanding of the signaling pathways operative in the glioma response to tamoxifen might allow the development of strategies that improve the antitumor efficacy of tamoxifen and/or the development of new and more effective agents that exploit similar antitumor mechanisms.

We first set out to explore whether the cytotoxic effects of tamoxifen on gliomas were specific to tamoxifen or whether other SERMs could exert similar effects. Our demonstration that CC-8490 induces apoptosis in human glioma cells demonstrates for the first time that a compound with SERM activity, other than tamoxifen, has antiglioma activity. In fact, we have tested several other SERMs and found at least one with similar antiglioma activity (data not shown). Given that all of the agents evaluated in this work have dramatically different chemical structures (the only known common characteristic is being capable of binding the ER with high specificity), it seems likely that the “off-target” mechanism being used by these compounds in glioma cells involves a molecule(s) that is structurally similar to ER. Faslodex, a pure ER antagonist, did not have antiglioma activity, adding further support for our contention that the ER itself is not the target for tamoxifen and CC-8490 in glioma cells. To date, the immediate binding target for the cytotoxic effects of these compounds in glioma cells remains unknown.

We were interested in exploring pathways that might be involved in the SERM-induced apoptotic response. It has been reported previously that activation of the PKC pathways was involved in the antiproliferative effects of tamoxifen on glioma cells; however, we could not substantiate significant changes in PKC activity after exposure of various glioma cells to tamoxifen or CC-8490. By contrast, we did find that exposure of glioma cells to cytotoxic tamoxifen and CC-8490 resulted in a large intracellular calcium influx, a phenomenon not seen after exposure of glioma cells to noncytotoxic SERMs. Consistent with these data was our finding, by gene expression profiling and quantitative PCR, of the selective deregulated expression of two genes that encode proteins known to be involved in the cellular response to calcium stress (STC and DSCR1). Cytosolic calcium fluxes regulate many physical functions, including neurotransmission, secretion, contraction, differentiation, cell growth, and cell death (15, 16). Elevation of cytosolic calcium, through either depletion of intracellular calcium stores or influx from the extracellular medium, is crucial for apoptosis (17). Consistent with our data, calcium influx has been associated with glial cell death induced by various stimuli including nicotine, amyloid β-peptide, calcium ionophore, and tamoxifen (5, 18, 19). Thus, it is quite conceivable that calcium may be an important second messenger for SERM-induced glioma cell apoptosis.

We used cDNA microarray gene expression profiling in an attempt to find other pathways that might be involved in the apoptotic response of glioma cells to tamoxifen and CC-8490. We identified 33 genes that were specifically and highly deregulated by both CC-8490 and tamoxifen (Table 1). Half of these responsive genes are apoptosis- and/or proliferation-related genes, including NF-κB1, IEX-1, SOD2, IL6, IL8, CDC2, GADD45A, and DDIT3. Some of these genes, however, may be involved in the general process of apoptosis, regardless of the initiating stimulus. Thus, we attempted to find a set of genes unique to CC-8490– and tamoxifen–induced cell death by identifying and then removing genes that are generally deregulated after exposure to other apoptosis-inducing stimuli such as DNA damage (BCNU), spindle disruption (Taxol), or serum starvation. This analysis resulted in a set of 20 genes that were highly significantly deregulated after exposure to only tamoxifen and CC-8490 (Table 1). These genes included NF-κB1, IER3, and SOD2 (Table 1).

The fact that CC-8490– and tamoxifen–induced apoptosis selectively induced up-regulation of NF-κB1 attracted our attention by virtue of the importance of NF-κB1 in the regulation of cell death and survival (20–31). The potential relevance of NF-κB1 up-regulation is supported by the finding that several additional genes identified in our analyses as specific to CC-8490– and tamoxifen–induced apoptosis are known downstream targets of NF-κB, including IER-1 (32–34), SOD2 (35–37), IL6 (38–40), and ILS (41–43), findings confirmed by quantitative PCR. These observations led us to hypothesize that
NF-κB might be involved in some aspect of SERM-induced glioma cell apoptosis.

Antiapoptotic activities of NF-κB have been observed after specific stimuli including tumor necrosis factor (TNF)-α, ionizing radiation, and DNA-damaging agents (20–26). In addition, oncopgenic Ras-induced p53-independent apoptosis is suppressed by NF-κB activation (44). In vivo studies have also demonstrated the protective effect of NF-κB on TNF-α–induced apoptosis in tumor-bearing mice (45), whereas RelA knockout mice die at 15 days of gestation secondary to massive hepatocyte apoptosis (46). Under specific conditions, however, NF-κB can also function in a proapoptotic fashion (26–31). Thus, the role of NF-κB activation in cell death appears to be context dependent, being dictated by variables such as the types of external stimuli, the cell type, and the microenvironment of the cell (25).

To determine whether NF-κB plays a role as an attenuator or a promoter of SERM-induced apoptosis in glioma cells, NF-κB activity was inhibited by sodium salicylate before SERM exposure. Salicylates are NF-κB inhibitors that suppress NF-κB activation through blocking phosphorylation and subsequent degradation of IκBα proteins (47, 48). Salicylates had no effect on glioma cell viability, although NF-κB activity was significantly diminished in the treated cells. In accordance with the hypothesis that NF-κB protects glioma cells from toxic stimuli, pretreatment of glioma cells with salicylates significantly enhanced SERM-induced apoptosis. Thus, it appears that NF-κB is induced in the glioma cells as a partial protective or stress response to the proapoptotic stimulus after exposure to tamoxifen and CC-8490. When the protective action of NF-κB is suppressed, however, the balance is even more tilted toward a proapoptotic effect from tamoxifen and CC-8490.
ptotic signal is not understood, although several of the SERM-selective induced genes are known to be NF-κB–induced genes involved with cell survival. For example, IEX-1 (also known as IER3) has been shown to protect cells from apoptosis induced by Fas or TNF-α through NF-κB activation (32) and can prevent apoptosis in activated T cells in vivo (34). Another antiapoptotic gene induced by tamoxifen and CC-8490 in the glioma cells was SOD2 (superoxide dismutase 2), another known NF-κB target gene (35–37). The correlation between SOD2 expression and increased resistance to cell death has been well established in several experimental systems (48–52). Among other SERM–up-regulated genes identified in our expression profiling were the cytokines IL6 and IL8, both known to be NF-κB–inducible genes (38–43). IL6 has been shown to protect against apoptosis induced by p53, Fas, and dexamethasone in tumor cells (53–56), whereas IL8 has also been identified as an apoptosis inhibitor in a number of different normal and tumor cell lines (57–59).

Taken together, our data suggest a model whereby selected agents with SERM activity induce a stress response in gliomas that is associated with an intracellular calcium influx, possibly leading to activation of a select set of genes and pathways. One predominant pathway activated by tamoxifen and CC-8490 exposure is the NF-κB pathway, leading to the up-regulation of a number of prosurvival/antiapoptotic genes. Accordingly, inhibition of the NF-κB pathway markedly sensitizes glioma cells to SERM-induced cytotoxicity, thereby identifying the NF-κB pathway as a compensatory glioma survival mechanism in response to tamoxifen and CC-8490 exposure.

These data provide additional insights into the antitumor effect of SERMs in non–ER-expressing glioma cells and provide a potential strategy for enhancing the efficacy of glioma treatment with SERMs. Although the serum concentrations of salicylates that would be necessary to inhibit NF-κB clinically would prove to be prohibitively toxic in vivo, small molecule inhibitors of NF-κB are being developed. Given the importance of the NF-κB pathway as a prosurvival mechanism after genotoxic insults, there is a concern that the use of such inhibitors in combination with traditional chemotherapy and/or radiation may cause significant toxicity to normal tissue. By contrast, SERMs display little normal tissue toxicity and thus are a theoretically attractive class of compounds to evaluate in combination with NF-κB inhibitors in future therapeutic trials of malignant gliomas.

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Agents with Selective Estrogen Receptor (ER) Modulator Activity Induce Apoptosis *In vitro* and *In vivo* in ER-Negative Glioma Cells

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